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# Antibacterial Activity in Marine algae *Eucheuma denticulatum* Against *Staphylococcus aureus* and *Streptococcus pyogenes*

[1,2]Nagi A. Al-Haj, [1]Nurmas I. Mashan, [1,3]Mariana N. Shamsudin,
[4]Habsah Mohamad, [5]Charles S. Vairappan and [1]Zamberi Sekawi
[1]Department of Microbiology and Parasitology, Faculty of Medicine and Health Sciences,
[2]Laboratory of Immunotherapeutic and Vaccine, Institute of Bioscience,
[3]Department of Marine Science and Aquaculture, Institute of Bioscience,
University of Putra Malaysia, 43400 Serdang, Selangor, Malaysia
[4]Department of Chemical Sciences, Faculty of Science and Technology,
Universiti of Malaysia Terengganu, 21030 Kuala Terengganu, Terengganu, Malaysia
[5]Laboratory of Natural Products Chemistry, Institute for Tropical Biology and Conservation,
University of Malaysia Sabah, 88999 Kota Kinabalu, Sabah, Malaysia

**Abstract:** The *in vitro* antibacterial activities of seaweed belong to *Euchema denticulatum* extract showed inhibitory activity only on gram positive organisms tested including *Staphylococcus aureus* and *Streptococcus pyogenes*, which were expressed in terms of minimum inhibitory concentration and minimum bactericidal concentration test. Thus, gram negative pathogens tested including *Escherichia coli*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* showed resistant phenotypic pattern to both extracts. Results of the present study confirmed the potential use of seaweed extract as a source of antibacterial compounds.

**Key words:** Seaweed, antimicrobial resistant, infectious disease, disc diffusion, minimal inhibitory concentrations, minimal bactericidal concentrations, Malaysia

## INTRODUCTION

The revolutionized therapy of infectious diseases by the use of antimicrobial drugs has certain limitations due to changing patterns of resistance in pathogens and side effects they produced. These limitations demand for improved pharmacokinetic properties, which necessitates continued research for the search of new antimicrobial compounds for the development of drugs. Seaweeds are used in traditional remedies in many parts of the world. The production of inhibitory substances by seaweed was noted as early as in 1917. Since then, numerous studies have been carried out to find and extract antimicrobial compounds from marine algae of *Eucheuma denticulatum* (Burman) Collins and Harvey, belongs to the family Solieriecеae, order Gigartinales, class Florideophyceae, division Rhodophyta. The former name was *Eucheuma spinosum* and spinosum is still used as commercial and trade name (Doty *et al*., 1987). *E. denticulatum* has a multiaxial structure and is composed of many rigid cylindrical branches narrowing to acute tips. The branches are usually densely covered with 1-8 mm long spine-like branchlets. Hence, follows the old name spinosum means bearer of spines. The spines may elongate into branches. The species is parenchymatous and a cross section of a branch reveals a dense core of very small, thick walled, rhizoidal cells at the centre of the medulla. *E. denticulatum* has, as most *Eucheuma* sp., small basal discs, only a few mm in diameter, for attaching itself to the substrate. The morphology may vary as a result of the environment in which, it grows. Brown, reddish and green morphophytes are all common in natural habitats and in cultivation (Fig. 1). The thalli of commercial plants vary from 50-100 g initial to about 1 kg in weight when they are harvested. But there have been reported plants of 56 kg fresh weight (Doty and Alvarez, 1973; Doty *et al*., 1987). Compounds from the seaweeds have antibacterial actions and some of these substances have potential use in mosquito control. Therefore, the present study is designed based on the phenotypic assay and genomic approach for determining antibacterial activity of *E. denticulatum* extracts. The investigation could scientifically proof the natural products to be potentially potent antibacterial agents.

**Corresponding Author:** Nagi AL-Haj, Laboratory of Immunotherapeutic and Vaccine, Institute of Bioscience, University of Putra Malaysia, 43400, Selangor, Malaysia

Fig. 1: *Euchema denticulatum*. Picture showed *E. denticulatum* type of red marine algae that were used in this study

## MATERIALS AND METHODS

**Bacteria sources:** The bacteria used in this study, *Staphylococcus aureus*, *Streptococcus pyogenes*, *Escherichia coli*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* were obtained from several hospitals in Malaysia. These isolates are represented in Table 1. All of the isolates were provided in the form of pure bacterial stock culture. Bacterial isolates were maintained at -20°C in Luria Bertani broth (Invitrogen Inc.) containing 15% ($v v^{-1}$) glycerol.

**Antibiotic susceptibility testing:** This antimicrobial susceptibility testing was determined by the disc diffusion method following the method recommended by the Clinical Laboratory Standard Institute (formerly known as NCCLS) (CLSI, 2005). In this study, only *Staphylococcus aureus* isolates were determined for antibiotic susceptibility to differentiate between Methicillin Resistant *S. aureus* (MRSA) and non-methicillin Resistant *S. aureus* (non-MRSA) isolates. The suspension of *S. aureus* culture was prepared equivalent to the MacFarland standard 0.5 and was lawned onto the Mueiller Hinton agar (Merck, Germany) plate to produce the bacteria field. Several antibiotic discs such as penicillin, vancomycin, erythromycin, gentamycin, mupirocin and methicillin (Difco Laboratories, Detroit, MI, USA) were put onto the bacteria field. The plate was incubated at 37°C and the zone of inhibition is observed after 24-48 h.

**Disc diffusion test:** Whatman paper No.1 filter paper was used to make sterile discs in order to screen for the antibacterial activity of *Euchema denticulatum*. This filter paper was punctured to the shape of commercial antibiotic disc and discs were autoclaved at 121°C for 15 min. The methanol extract of either *E. denticulatum* was solubilised in the 60% methanol. The suspension of bacteria culture was prepared according to the MacFarland standard 0.5 and was lawned onto the Mueiller Hinton agar plate to produce the bacteria field. A sterile of punctured filter papers was placed on the bacteria field by a sterile forcep and the solubilised extract then was pipetted out onto the surface of filter paper on the bacteria field. Sixty percent methanol was used as a negative control while the commercial antibiotic discs were used as a positive and negative control. Finally, the plate was incubated at 37°C and the zone of inhibition is observed after 24-48 h.

Table 1: Sources of bacteria isolates used in the study

| Isolate No. | Bacteria | Isolate Identity | Hospital |
|---|---|---|---|
| 1 | *Staphylococcus aureus* | STR 1 | UMMC |
| 2 | *S. aureus* | STR 2 | UMMC |
| 3 | *S. aureus* | STR 5 | UMMC |
| 4 | *S. aureus* | STR 7 | UMMC |
| 5 | *S. aureus* | STR 8 | UMMC |
| 6 | *S. aureus* | STR 9 | UMMC |
| 7 | *S. aureus* | STR 10 | UMMC |
| 8 | *S. aureus* | N1 | Kuantan |
| 9 | *S. aureus* | N2 | Kuantan |
| 10 | *S. aureus* | N3 | Kuantan |
| 11 | *S. aureus* | N5 | Kuantan |
| 12 | *S. aureus* | N6 | Kuantan |
| 13 | *S. aureus* | N7 | Kuantan |
| 14 | *S. aureus* | N8 | Kuantan |
| 15 | *S. aureus* | 2 | Miri |
| 16 | *S. aureus* | 4 | Miri |
| 17 | *S. aureus* | 8 | Miri |
| 18 | *S. aureus* | 10 | Miri |
| 19 | *S. aureus* | 16 | Miri |
| 20 | *S. aureus* | 18 | Miri |
| 21 | *S. aureus* | 20 | Miri |
| 22 | *Streptococcus pyogenes* | SP1 | Seremban |
| 23 | *Escherichia coli* | EC1 | Batu Pahat |
| 24 | *Klebsiella pneumoniae* | KP1 | Seremban |
| 25 | *Pseudomonas eruginosa* | PA1 | UMMC |

**Minimal Inhibitory Concentration Test (MIC) and Minimal Bactericidal Concentration Test (MBC):** The sensitivity of bacteria to *E. denticulatum* methanol extracts can be measured by using a tube dilution technique, which determines the MIC ad MBC of seaweed used in this study *in vitro*. These tests were done to determine the lowest concentration of either *E. denticulatum* extract, where it can show the bactericidal and bacteriostatic effect. The test was performed in 96-well microtitre plates, so that several replicates of each sample can be run. All isolates were grown until the concentration is equal to 0.5 MacFarland standard at 37°C and diluted in Mueller Hinton Broth (MHB; Difco Laboratories, Detroit, USA) supplemented with 2% NaCl (Thornsberry and McDougas, 1983) to a concentration of 50 mg $mL^{-1}$ for *E. denticulatum* and serial two-fold dilutions was made. Then, the suspension of *S. aureus* culture was added into the 96 well microtitre plates

containing diluted sample of *E. denticulatum* extract. Finally, the 96 well microtitre plates containing diluted sample of *E. denticulatum* and bacteria was then incubated overnight at 37°C with constant shaking on the shaker. On the next day, the diluted sample of *S. aureus-E. denticulatum* in the 96-well microtitre plates were plated out onto the Mueiller-Hinton agar (Merck, Germany) plate according to the concentration of either *E. denticulatum* extract. The plate was incubated at 37°C for 24 h in the incubator. Finally, the number of bacteria colonies developed on each agar plates was counted.

## RESULTS

**Disc diffusion test:** In this test, filter paper disc impregnated with methanol extract of *Euchema denticulatum* similar clear inhibition zone around discs are seen in the MRSA (Fig. 2 and 3) and lawn of non-MRSA isolates (Fig. 3 and 4). Clear inhibition zones are also seen in isolates lawned with *S pyogenes* (Fig. 5). Plates lawned with gram negative bacteria namely *E. coli* (Fig. 6), *P. aeruginosa* (Fig. 7) and *K. pneumoniae* (Fig. 8) showed no inhibition zone around discs.The results of inhibition and no inhibition zones represented for tests that were performed twice.


Fig. 2: Disc susceptibility testing of *Euchema denticulatum* on *Staphylococcus aureus* (STR5). Clear inhibition zone is seen around the filter paper disc impregnated with extract. Inhibition zone around vancomycin disc (positive control) also is seen

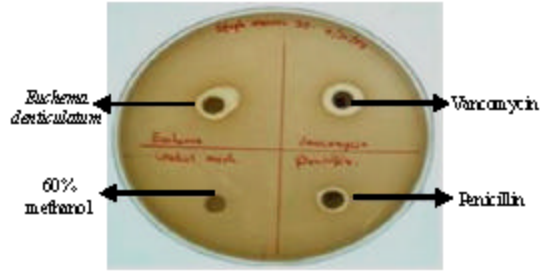


Fig. 3: Disc susceptibility testing of *Euchema denticulatum* on *Staphylococcus aureus* (20). Clear inhibition zone is seen around the filter paper disc impregnated with extract. Small inhibition zone around vancomycin disc (positive control) also is seen

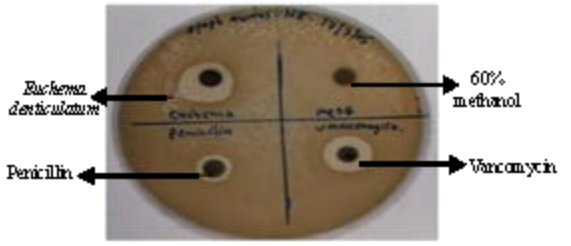


Fig. 4: Disc susceptibility testing of *Euchema denticulatum* on *Staphylococcus aureus* (N8). Clear inhibition zone is seen around the filter paper disc impregnated with extract. Small inhibition zone around vancomycin disc (positive control) also is seen

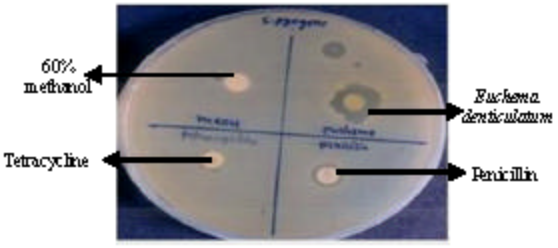


Fig. 5: Disc susceptibility testing of *Euchema denticulatum* on *Staphylococcus pyogenes*. Clear inhibition zone is seen around the filter paper disc impregnated with extract. Small inhibition zone around penicillin disc (positive control) also is seen

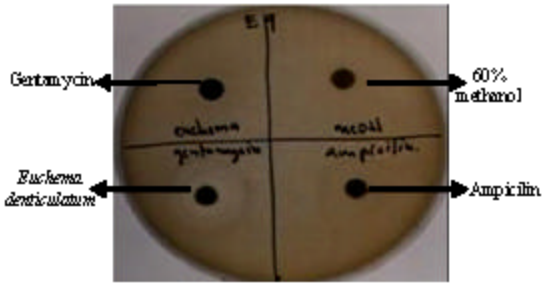


Fig. 6: Disc susceptibility testing of *Euchema denticulatum* on *Escherichia coli*. No inhibition zone is seen around the filter paper disc impregnated with extract. Only inhibition zone around gentamycin disc (positive control) is seen

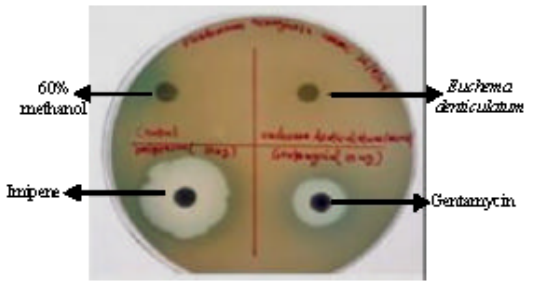


Fig. 7: Disc susceptibility testing of *Euchema denticulatum* on *Pseudomonas aeruginosa*. No inhibition zone is seen around the filter paper disc impregnated with extract. Only inhibition zone around gentamycin and imipenem disc (positive control) are seen

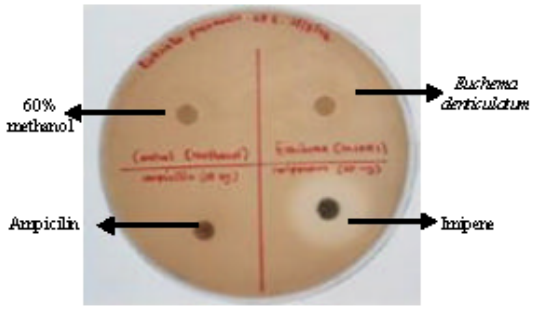


Fig. 8: Disc susceptibility testing of *Euchema denticulatum* on *Klebsiella pneumoniae*. No inhibition zone is seen around the filter paper disc impregnated with extract. Only inhibition zone around imipenem disc (positive control) is seen

**Minimal Inhibitory Concentration Test (MIC) and Minimal Bactericidal Concentration Test (MBC):** Minimal Inhibitory Concentration (MIC) and Minimal Bactericidal Concentration (MBC) determined based on the lowest *Euchema denticulatum* extract concentration reducing colonial growth or killing all bacteria cells. The MIC and MBC tested for *E. denticulatum* are for 2 isolates of MRSA and 2 isolates of non-MRSA. MIC and MBC of *E. denticulatum* extract against *S. aureus* showed the comparison between the numbers of colonies growth in different concentration of *E. denticulatum* (Fig. 9 and 10) extracts. According to the Table 1, methanol extract of *E. denticulatum* showed strong activity irrespective of MRSA and non-MRSA isolates, the MIC and MBC level for *E. denticulatum* extract are 20 and 40 mg $mL^{-1}$ for MRSA isolates and 8.75 and 17.5 mg $mL^{-1}$ for non-MRSA isolates, respectively.

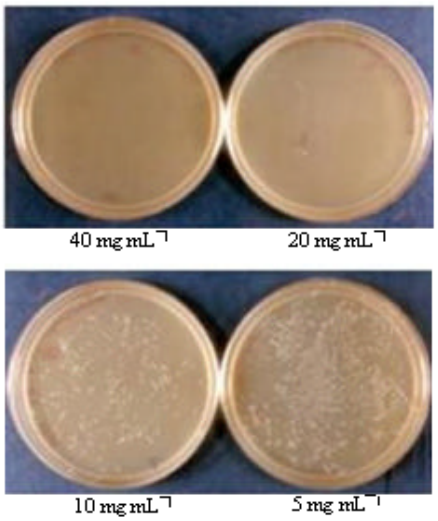


Fig. 9: MIC and MBC test for MRSA isolate with *Euchema denticulatum* extract. The reducing of colonial growth of STRS (MRSA) isolation in different concentrations of *E. denticulatum* methanol extract

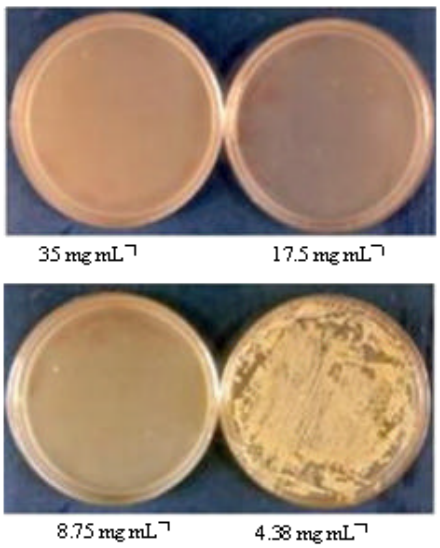


Fig. 10: MIC and MBC test for non MRSA isolate with *Euchema denticulatum* extract. The reducing of colonial growth of N8 (non-MRSA) isolation in different concentrations of *E. denticulatum* methanol extract

## DISCUSSION

Seaweeds or algae are a eukaryotic organism (Michael *et al*., 2002) that lives in salty water in the ocean and is recognized as a potential source of bioactive

natural products. They contain compounds ranging from sterols, terpenoids to brominated phenolic, which show bioactivity against microorganisms (Wong *et al*., 1994). Polysaccharide derived from red algae was used widely since the 19th century as agar, a type of gelling agent (Michael *et al*., 2002). Sulfated polysaccharides that extracted from red algae has anticoagulant activities (Caceres *et al*., 2000; Farias *et al*., 2000; Yamada *et al*., 2000), anti-thrombotic activities and antiviral effects against herpes simplex virus (Caceres *et al*., 2000) and Human Immunodeficiency Virus (HIV) (Yamada *et al*., 2000). The main objective of this study, is to determine the antibacterial activity of local seaweed from the Malaysian's ocean, *Euchema denticulatum* against *Staphylococcus aureus*, *Escherichia coli*, *Streptococcus pyogenes*, *Pseudomonas aeruginosa* and *Klebsiella pneumoniae*. The activity of the seaweed's methanol extract will lead to a discovery of antimicrobial agents derived from these natural products as an alternative to substitute the existing antibiotic, which is already resistant to the pathogens worldwide, especially in the treatment of Methicillin-Resistant *Staphylococcus aureus* (MRSA). The research approaches used in this study was bioassays or preliminary screening, which included the screening of antibacterial activity through disc diffusion test and minimal inhibitory concentration test. In the present study, several significant findings were found whereby the methanol extract of *Euchema denticulatum*, exhibited the inhibitory activity against *Staphylococcus aureus* and *Streptococcus pyogenes*.

The inhibitory activities of both extracts were indicated both in MRSA and non-MRSA isolates tested. The methanol extract of *E. denticulatum* exhibited more intense activity against MRSA and non-MRSA isolates. The current study revealed that the extract inhibited a MRSA isolate, STR9 and a non-MRSA isolate, N8, with zones of about 3 and 2 mm more than the inhibition zones around the vancomycin disc, respectively. The isolate, STR5, was also inhibited by the extract but with a smaller inhibition zone, which is 1 mm less than the zone around the vancomycin disc. The extract can be considered valuable as an alternative in the treatment of MRSA and non-MRSA infection substituting penicillin, since this extract inhibited both MRSA and non-MRSA with inhibition zones of 6 and 1 mm more than the inhibition zone developed around penicillin disc. In addition, seaweed extracts also indicated significant inhibitory activity against *S. pyogenes* isolates. However, further research on *S. pyogenes* isolates, was not performed, since this current study is focused on the inhibitory activity of both extracts against *S. aureus*. Despite of the significant activity of both seaweed extract against MRSA and non-MRSA isolates, not all bacteria respond equally to the given extracts especially for gram negative isolates. From the literature by Ballantine *et al*. (1987) and Reichelt and Borowitzka (1984), the higher frequency of activity against gram positive bacteria has been observed in most of the surveys of antimicrobial activities from seaweeds if compared with activity against gram negative bacteria.

Thus, this present study also showed the same inhibitory pattern whereby the antibacterial activity of *E. denticulatum* extracts were only observed in gram positive bacteria and no inhibitory activity against tested gram negative bacteria such as *Escherichia coli*, *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* were indicated. According to Michael *et al*. (2002), there is several reasons why certain bacteria may develop resistant to certain antimicrobial agents that may explain the findings of the current study on gram negative bacteria. Absence of the target structure in the bacteria, the ability of the bacteria to alter the structure of the extracts to an inactive form and the genetic changes in the bacteria could be some reasons for not inhibiting the bacterial growth. In addition, the alteration may occur in a metabolic pathway that the extracts will block, which may contribute to the resistant phenotype of the bacteria to both seaweeds extracts. In bacteria, the important targets of antimicrobial action are the cell wall, the cytoplasmic membrane, the biosynthetic processes of protein synthesis and nucleic acid synthesis. The cell wall combining with additional layers and outer membrane in gram negative bacteria such as *P. aeruginosa* limits the penetration of both extracts.

Sometimes, the bacteria may be modifying either one of the targets so that the extracts cannot take action. In *P. aeruginosa*, production of alginate slime may protect their cells from therapeutic levels of antimicrobial agents. However, even though the methanol extract of *E. denticulatum* can be classified into the narrow spectrum antimicrobial agents, which acts on only a single group of organism, both extracts are still quite valuable for the control of microorganisms especially, MRSA and non-MRSA that fail to respond to available antibiotics. A fundamental concept of *in vitro* susceptibility testing is the measurement of Minimum Inhibitory Concentration (MIC) and Minimal Bactericidal Concentration (MBC) of *Euchema denticulatum* extract that exhibit their antibacterial activity against Methicillin Resistant *Staphylococcus aureus* (MRSA) and non-Methicillin Resistant *Staphylococcus aureus* (non-MRSA). Both methods were chosen in order to point out if there might be some significant difference between the lowest concentration of both extracts required to inhibit growth of *S. aureus* and the lowest concentration at which both

extracts can kill 99.9% of the initial *S. aureus* inoculums. These were indicated by an absence of visible turbidity of the Mueiller-Hinton broth and an absence of bacterial colonies on the Muieller-Hinton agar. Since, the bactericidal and bacteriostatic terminology originates from either the antibacterial's mechanism is based on inhibiting cell wall formation (bactericidal) or inhibiting bacterial metabolism or ribosomal protein synthesis (bacteriostatic) (Zarakolu *et al*., 1999), the idea is that if cell wall formation is blocked, the organisms will lyse and perish, but if metabolism or protein synthesis is blocked, the organisms merely slow down. While, this is true to some degree, bactericidal or bacteriostatic outcomes are dependent on the concentration of the antibacterial agent as well. A low dose of a bactericidal antibacterial agent may only inhibit bacterial growth, while a high dose of a bacteriostatic antibacterial agent will be bactericidal. Additionally, according to Zarakolu *et al*. (1999), organisms which are not proliferating may not be significantly affected by anti-cell wall antibiotics, in which case anti-ribosomal antibiotics would be more effective.

Therefore, in this study, MIC would be taken as the reference point for the study designations susceptible or resistant, while MBC determinations would be more certain for the prediction of susceptibility in the cases that required bactericidal therapy rather than bacteriostatic theraphy. MBC and MIC tests were done carefully since it is easy affected by the nature of the bacteria used, the inoculums size and the composition of the culture medium, the incubation time and the conditions of incubation, such as temperature, pH and aeration. In the present study, the reading of MIC and MBC, which were based on the number of colonies growing on the Mueiller-Hinton agar is constant for both seaweeds after repeated twice. The present study revealed that methanol extract of *E. denticulatum* inhibited or reduced the growth of *S. aureus* isolates. Since, the inhibitory effect of *E. denticulatum* extract to *S. aureus* isolates is greater if compared to the inhibitory effect of *G. changii* extract to *S. aureus* through the disc diffusion test.

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